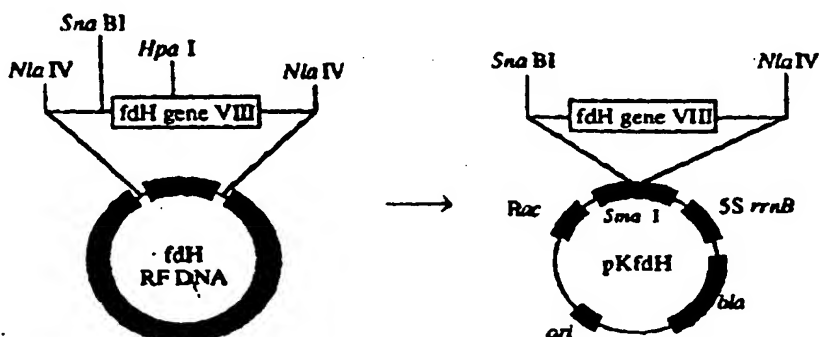




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(54) Title: ENGINEERED BACTERIOPHAGES AND VACCINES CONTAINING THEM



## (57) Abstract

A filamentous bacteriophage is engineered to display T-cell epitopes and B-cell epitopes and/or a peptide capable of eliciting HIV-neutralising antibodies. Effective immunogenicity is observed.

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ENGINEERED BACTERIOPHAGES AND VACCINES CONTAINING THEMField of the Invention

This invention relates to engineered bacteriophage and vaccines containing them.

5 Background of the Invention

The treatment of infectious diseases remains a considerable problem. Major efforts are being made to develop new and/or improved vaccines. For a variety of reasons, progress is slow, but the discovery that  
10 protective immunity can be obtained by administration of only one of the constituent proteins of a pathogen, and in some cases only a fraction of protein, has offered new hope in the production of vaccines.

Foot-and-mouth disease virus (FMDV) belongs to the  
15 picornaviridae, a family of positive-sense RNA viruses. FMDV remains a major scourge of live-stock in several continents. The control of this disease is either by slaughter, in those countries where FMDV does not normally occur, or by vaccination in endemic areas. The vaccines  
20 that are currently in use are made from inactivated virus, which has been grown in tissue culture cells. This vaccine has proved to be very effective in controlling the disease, but there are many problems associated with handling large quantities of this highly infectious agent, and in ensuring  
25 its complete inactivation. Moreover, these vaccines are difficult to administer in remote regions, and severe economic losses due to the disease still occur.

Another good example of a widespread disease, and which has eluded immunologists for many years, is malaria.  
30 The major human malarial pathogen, Plasmodium falciparum, has a complex life-cycle and an ever-changing repertoire of surface antigens which allows highly effective evasion of the human immune-system. Numerous attempts have been made to control the disease but no satisfactory vaccine,  
35 suitable for widespread administration, has been obtained.

Irradiated sporozoites have been used to immunize man, and protection against malaria has been achieved. However,

in this type of vaccine, both sexual and asexual forms of the parasite are required; sporozoites must, therefore, be prepared either from the salivary glands of infected mosquitoes or from cultured red blood cells. The former  
5 yields very small quantities, and the latter is a very expensive process.

The protective response to irradiated sporozoites has been localized to a protein (called the circumsporozoite protein) which is expressed on the surface of the organism  
10 and which does not mutate between generations. The most striking feature of this polypeptide chain is a large central repeat of the amino-acid sequence NANP (SEQ. ID No. 1), sometimes interspersed with the sequence NDVP (SEQ. ID No. 2). Further investigation has shown that twelve amino-  
15 acid units of this repeat sequence are sufficient for the production of antibodies reactive against the whole molecule. A wide variety of peptides, of twelve amino-acids or more, containing the repeat region of the circumsporozoite protein have been used to inoculate  
20 rabbits and mice; see, for example, Young et al., Science 228 (1985) 985. Immunogenic responses have been obtained, but these are limited because oligopeptides do not generally provoke a humoral response.

Small peptides are poorly immunogenic, because they  
25 are missed by the cells of the immune-system or interact weakly with the cells they do meet. In addition, small peptides are excreted by the kidneys and, therefore, have only a short life-span in the blood-stream. In animal systems, some of these problems may be overcome by  
30 administering Freund's Complete Adjuvant (FCA) or aluminium hydroxide with the peptide, but both of these adjuvants are highly toxic and cannot be administered to humans. Peptides may be made more immunogenic if they are linked to a large carrier system after they have been synthesized,  
35 but this adds both to the complexity and expense of vaccine production.

To circumvent some of the problems associated with using small peptides as antigens, a different approach for vaccine production has been investigated. The genetic material of a virus is manipulated such that a foreign antigenic peptide is expressed on the surface of the virion. A DNA sequence which codes for the antigen is inserted into one of the coat protein genes; this may be a complex procedure, since many viruses contain RNA rather than DNA. For example, an antigenic epitope from polio virus type III has been inserted into the coat protein of tobacco mosaic virus; see Haynes *et al.*, *Biotechnology* 4 (1986) 637. A hybrid coat protein was produced and over-expressed in *E. coli* and, after purification, it could be assembled into virus-type rods. The hybrid TMV particles, injected into rats, were able to stimulate the production of antibodies which could neutralize polio virus. Unfortunately, this response was only obtained when FCA was employed.

Antibodies have also been expressed on the surface of attenuated animal viruses such as vaccinia, herpes and polio; see Dimmock *et al* (ed.), *Foc. Gen. Microbiol. Symp.* 45, "Control of Virus Diseases", pub. Cambridge Univ. Press (1990). There are several problems associated with using animal viruses as vaccines: they are complex and their genetic material may be difficult to manipulate; they are difficult to prepare in large amounts and hence are expensive; they can sometimes revert to pathogenesis or exhibit some other form of toxicity.

Filamentous bacteriophages contain a single-stranded DNA genome which is encased within a protein sheath. The DNA becomes double-stranded after infection of the host strain, *Escherichia coli*, and both double- and single-stranded forms of DNA may be easily purified and manipulated (the virus M13, which is commonly used as a vector for site-directed mutagenesis and sequencing, is a filamentous bacteriophage). The genome encodes ten proteins, five of which are found in the mature phage

particle. Gene VIII encodes the major coat protein which forms the tubular structure of the phage capsid, genes VII and IX encode minor coat proteins which are located at one end of the phage particle, and genes III and VI encode  
5 minor coat proteins positioned at the other end. A virus particle contains about five copies of each of the minor coat proteins.

The product of gene III is a large protein involved in infection of the host cell. Chemically-synthesized DNA  
10 cassettes have been cloned into gene III and recombinant proteins have been produced; see de la Cruz et al., J. Biol. Chem. 9 (1988) 4318. A number of recombinant proteins have been incorporated into viral particles and these were used to inoculate rabbits and mice, but in no  
15 case did the immune-system respond with sufficient vigour to confer immunity. Although phages engineered in this way do not seem to be suitable for use as vaccines (since large and potentially dangerous doses would be required to elicit an effective response), this system is being developed for  
20 production of "epitope-libraries" used, for example, to investigate the specificity of antibodies; see Scott & Smith, Science 249 (1990) 386.

Il'ichev et al., Doklady Akademii Nauk SSR 307(2) (July 1989) 481-3, disclose phage M13 having a foreign peptide,  
25 5 amino-acids long, incorporated into the major envelope protein. A "substantial effect on the infectiousness of the phage" is reported; this effect is actually a considerable decrease, to about 10%, of the reinfection potential. No other properties of the engineered virus  
30 were reported.

Rowitsch et al., J. Mol. Biol. 204 (1988) 663-674, disclose hybrid phage assembly.

#### Summary of the Invention

According to the present invention, a filamentous  
35 bacteriophage includes, in at least a proportion of its major coat protein, a foreign peptide of 6 or more amino-acids that is capable of eliciting a biological response.

The present invention is based on the realisation that the failure of the gene III fusion proteins in vaccine production is almost certainly due to the low number of proteins found in each phage and/or to the masking of the epitope by the bulk virus structure. The major coat protein (the product of gene VIII), however, is found as about 2500 copies per virus, and peptides having an immunogenic effect have now been inserted into this protein. Moreover, they are expressed in a region of the coat protein known to be exposed on the surface of the virus. As indicated below, the coat protein containing the foreign peptide may desirably be present in balance with unmodified (wild-type) virus coat protein.

#### Description of the Drawings

Figure 1 is a schematic view of a hybrid filamentous bacteriophage. It shows a gene III protein 1, a coat protein with epitope 2, a wild-type coat protein 3, and an epitope 4 that is too large for normal packing of subunits.

Figure 2 shows the construction of plasmid pKfdH. The process involves isolating the NlaIV fragment and then the SnaBI/NlaIV fragment, followed by ligation into SmaI/phosphatase-treated pKK223-3.

#### Description of the Invention

A bacteriophage of the invention includes a foreign peptide: the peptide may be antigenic, e.g. any that raises a desired immunological response, e.g. against a form of the malaria parasite or against another virus such as foot-and-mouth disease virus. It may already be known for this purpose. Its length should be sufficient to raise the response but insufficient to modify the bacteriophage's properties undesirably or to prevent its incorporation, e.g. at least 9 amino-acids.

A bacteriophage of the invention is preferably immunogenic, and is suitable for use in vaccines, and generally as a therapeutic/diagnostic product. Therefore, products of the invention may be formulated with any

suitable physiologically-acceptable diluent or carrier, to prepare a vaccine composition.

In addition, by using two or more compatible vectors to carry suitably engineered coat protein genes, two or more differently modified coat proteins can be assembled into the same virion, to enable more than one peptide to be displayed simultaneously. This can lead to the generation of multi-shot vaccines and to the generation of more complex structures on the surface of the virion with correspondingly complex biological properties.

A potential disadvantage of this or other viruses as vaccines is that they contain DNA, which may be regarded as undesirable for injection into the animal being protected. With the filamentous bacteriophage carrier described here, the DNA within the virion may be selectively degraded by dialysis at acid pH before injection.

In an alternative embodiment of the invention, the foreign peptide is an agonist/antagonist of a cell receptor (e.g. a biologically-active peptide such as a hormone, releasing factor or growth factor) or an inhibitor of an enzyme (e.g. an inhibitor of renin or the AIDS virus proteinase) or an enzyme (phage enzyme) or an antibody (phage antibody) or any other protein or peptide with a desired biological activity. For some purposes, the peptide may include a non-metabolisable component. Bacteriophages including a variety of such peptides can be used to screen for preferred structure and activity. In addition, the hybrid phage system has other broad applications. By inserting varied or random DNA sequences to encode the peptides to be displayed, an infinite number of peptides can be expressed on the surface of the virion, thus creating "epitope libraries".

Epitope libraries allow large numbers (sometimes tens of millions) of small peptides to be surveyed for any desirable property, e.g. tight binding to an antibody, receptor or other binding protein. For example, while antibodies can be purified from the serum of a patient with



an auto-immune disease, the target of the antibodies is often unknown. An epitope library can be used to find the antigenic target of the antibodies, hence identifying the physiological site of the disease and suggesting a route for therapeutic procedures. It may also be possible to discover peptides which mimic the biologically-active regions of hormones, cytokines, enzyme substrates, neuropeptides and other biomolecules. Peptides which could either replace or alter the action of the natural ligands would be powerful candidates for drug development. The hybrid phage allows the number of copies of a peptide displayed on each viral particle to be controlled within wide limits, and can confer great sensitivity.

The major coat protein of the filamentous bacteriophage fd is encoded by gene VIII. The protein is synthesized as a procoat which contains a 23 amino-acid leader-peptide attached to the N-terminus of the mature protein. After synthesis, the procoat protein is rapidly inserted into the inner-membrane of E. coli where it is processed to leave the 50 amino-acid mature coat protein spanning the membrane. This protein has three domains, a hydrophobic membrane-spanning domain, a positively-charged C-terminal domain which faces into the cytoplasm of the cell, and a negatively-charged N-terminal domain which extends into the periplasm. During assembly of virus articles, the coat protein subunits are pulled out of the cell membrane and become arranged in a helical array around the viral DNA. In the viral particle, the C-terminal region of the protein subunits faces inwards towards the DNA such that the positively-charged residues may neutralize the negative charges of the sugar-phosphate backbone. The N-terminal domain, by contrast, is on the outer surface of the particle where it is exposed to the environment. Nuclear magnetic resonance studies have indicated that this region is flexible both in the membrane-bound form of the protein and when presented on the outside of the phage particle. This region is also

known to be highly antigenic, antibodies prepared against whole phage particles being directed almost solely towards this domain of the coat protein. In this invention, peptides are attached to the outside of the phage coat protein, to give extended coat proteins that successfully assemble into viral particles and elicit a strong immune-response.

Therefore, in order to prepare an embodiment of the invention, epitopes are inserted into the N-terminal domain of the major coat protein of bacteriophage fd, such that they decorate the outside of an assembled phage particle. This problem may be approached entirely at the DNA level, by engineering a restriction enzyme recognition site into gene VIII, and allowing insertion of oligonucleotide cassettes, encoding the desired epitope, directly into the phage genome. In order to study this problem in a controlled manner, however, it was preferred to separate the synthesis of any hybrid coat proteins from the assembly of phage particles. This allows the cloning and expression of novel coat proteins, and the membrane-insertion and packaging stages of phage assembly, to be studied as independent entities.

In order to allow independent expression of the fd coat protein gene, gene VIII may be subcloned into a controllable expression vector such as pKK223-3 which uses the tac promoter. However, the wild-type gene VIII does not contain a suitable restriction enzyme site for the insertion of oligonucleotide cassettes (which would be used to encode the epitopes). Therefore, before the gene is subcloned, a suitable site may be introduced by means of site-directed mutagenesis. By way of example, this site was blunt-ended (to allow "in-frame" insertion of DNA), was positioned between the third and fourth codons of the mature coat protein (in order to minimize disruption of the leader-peptidase recognition sequence in the procoat) and was recognised by HpaI which does not have any recognition sites in pKK223-3, thus providing a unique cloning site.

In Preparing a hybrid bacteriophage of the invention, an intermediate product is a plasmid encoding the modification. In order to prevent the loss of such a plasmid, it is preferred that the engineered coat protein is transplanted into a phage, phagemid or other replicon, with a packaging signal. The recombinant gene can then be packaged and selected for further replication.

The following Examples illustrate the preparation and utility of products of this invention. With the exception of the methods A, B and C described below, all experimental procedures are as described by Greenwood et al, J. Mol. Biol. 220 (1991) 821-827; see also Rowitsch et al (1988), supra.

A Polyacrylamide gel electrophoresis using the tricine buffer system

Proteins were separated on denaturing polyacrylamide gels composed of: 49.5% (w/v) acrylamide/ 0.5% (w/v) bisacrylamide (7 ml), 3 M Tris HCl, pH 8.4 (7 ml), glycerol (4.2 ml) and H<sub>2</sub>O (2.8 ml), set with 25% (w/v) ammonium persulphate (200 µl) and TEMED (20 µl). The stacking gel contained: 49.5% (w/v) acrylamide/ 0.5% (w/v) bisacrylamide (1 ml), 3 M Tris HCl, pH 8.4 (2.5 ml) and H<sub>2</sub>O (6.8 ml), set with 25% (w/v) ammonium persulphate (100 µl) and TEMED (10 µl). The gel cassette measured 17 cm x 17 cm and was 1 cm thick.

Samples were loaded in 4 times their volume of denaturing buffer, i.e. DTT (154 mg), 10% (w/v) SDS (2 ml), glycerol (1 ml), 1 M Tris HCl, pH 6.8 (170 µl), H<sub>2</sub>O (1.63 ml), 0.2 % (w/v) Bromophenol blue in ethanol (200 µl). Samples were heated at 100°C for 2 min before loading onto gels. The running buffer contained: 1.8% (w/v) Tricine, 1.2% (w/v) Tris base and 1% (w/v) SDS.

Gels were run overnight at 100 V or during the day at 250 V until the bromophenol blue dye front reached the bottom.

**B Electroblotting of proteins onto PVDF membrane**

Proteins were separated using tricine-PAGE as described in Method A with the addition of 2 mM mercaptoacetic acid in the upper electrode buffer (used to  
5 scavenge N-blocking free radicals). When the gel had run to completion it was soaked in transfer buffer (25 mM Tris base, 190 mM glycine, 10% (v/v) MeOH) for 10 min. The PVDF membrane was prepared by immersion in MeOH for 10 s followed by equilibration in transfer buffer for 5 min. The  
10 proteins were electroblotted onto the PVDF membrane using a Biorad transblot apparatus. Transfer was carried out at 150 mA for 3-16 h with cooling to 16°C. When transfer was complete the PVDF was immediately soaked in water for 10 min with shaking. The membrane was rinsed in water and was  
15 allowed to air-dry thoroughly.

**C Detection of proteins on Western Blots using alkaline phosphatase**

Proteins were electrophoretically transferred onto nitrocellulose. The membrane was soaked in TBST buffer (10  
20 mM Tris HCl, pH 8.0, 0.9% (w/v) NaCl, 0.05% (v/v) Tween 20) for 10 min, followed by the same buffer containing 1% (w/v) BSA for 20 min. The membrane was washed with TBST (without BSA) for 10 min. The membrane was soaked in TBST containing antibody directed against bacteriophage fd or  
25 the hybrid bacteriophage (1 µl antibodies/1 ml TBST) for 30 min and was then washed in TBST 3 times (10 min per wash). After washing, goat anti-rabbit IgG-alkaline phosphatase-conjugate was added (1 µl antibodies/1 ml TBST) and incubation was continued for 30 min. The membrane was  
30 washed 3 times as before and the blot was developed in AP buffer (100 mM Tris HCl, pH 9.0, 100 mM NaCl, 5 mM MgCl<sub>2</sub>) containing about 1.6 mg nitro blue tetrazolium and about 0.8 mg 5-bromo-4-chloro-3-indolyl phosphate. Both of the substrates could be prepared as stocks in water shortly  
35 before use.

Example 1

## 1.1 Site-directed mutagenesis and subcloning of fd gene VIII

An 18mer oligonucleotide (SEQ ID No. 3) was synthesized on a Biosearch 8600 machine using phosphoramidite chemistry, and was purified by polyacrylamide gel electrophoresis. Single-stranded DNA template was prepared from wild-type fd and mutagenesis was performed using the phosphorothioate method and available in kit form from Amersham International plc. A number of bacteriophages were selected and sequenced using a second oligonucleotide (SEQ ID No. 4, synthesized and prepared as above) which binds towards the 3'-end of gene VIII. A phage was isolated which bore the correct mutation and was designated fdH.

Insertion of the HpaI site led to a change in the amino-acid sequence of the mature coat protein (SEQ ID No. 5) from Gly-Asp at positions 3 and 4 to Val-Asn (SEQ ID No. 6). Such a change clearly did not significantly affect the membrane-insertion and processing of the procoat protein, nor the assembly of the coat protein into bacteriophage particles, since mutant phage were viable and could be propagated in yields comparable with the wild-type.

The scheme for subcloning the fdH gene VIII is shown in Figure 2. Double-stranded RF DNA of fdH was prepared and digested with the blunt-end cutter NlaIV to generate a number of fragments including a 292 bp fragment which contained gene VIII. This band was isolated by polyacrylamide gel electrophoresis and was further digested with a second blunt-end cutter, SnaBI, which removed 20 base pairs upstream of the gene (already known to interfere with efficient expression of the wild-type gene). The shortened fragment was again purified by polyacrylamide gel electrophoresis and was cloned into pKK223-3 which had been cut with SmaI and treated with calf intestine alkaline phosphatase. Recombinants which contained the fdH gene in

the correct orientation for expression were identified by restriction digests and were designated pKfdH.

## 1.2 Expression of the fdH gene

Expression of the fdH gene was tested in vivo using E. coli strain TG1 (lacIq) transformed with plasmid pKfdH. Transformed cells were grown in YT medium supplemented with 50  $\mu$ g ampicillin/ml at 37°C to an  $A_{600}$  value of approximately 0.3, and were then induced with IPTG (final concentration 250  $\mu$ M). At various times after induction samples of cells were removed, collected by centrifugation, washed and analysed by means of polyacrylamide gel electrophoresis using the tricine buffer system (tricine-PAGE). Staining the gels with silver revealed that before induction there was no detectable coat protein produced; after induction, transformed cells contained increasing amounts of a protein which had an electrophoretic mobility similar to that of wild-type coat protein. In these in vivo experiments, the procoat protein is being inserted into the cell innermembrane and is being processed by leader-peptidase. In the case of the fdH protein, the mutations clearly had not interfered with either of these processes and there were large amounts of processed coat protein available in the cell membrane.

Therefore, the insertion of the HpaI site and the concomitant change in the primary structure of the procoat protein did not interfere with bacteriophage assembly. The mutated coat protein was successfully cloned into the controllable expression vector pKK223-3 where its synthesis could be induced at will. The procoat protein synthesized from the plasmid was also competent for membrane-insertion and was correctly processed.

Although it was perhaps unsurprising that the insertion of the HpaI site into fd gene VIII had little effect on the membrane-insertion and processing of the slightly altered procoat protein, the addition of a number of extra amino-acids into this small protein was a novel and more dramatic procedure, and the effects on membrane-

insertion could not be predicted. Failure at this stage would obviously prohibit assembly of the fusion coat proteins into viral particles.

5 The second stage of the work, therefore, was to insert an oligonucleotide cassette into the HpaI site and to investigate the effects on the membrane-insertion and processing of the resulting procoat protein. The chosen epitope (SEQ ID No. 7) was the repeat sequence of the Plasmodium falciparum coat protein (SEQ ID No. 1). This was known to elicit an immune response when administered as a synthetic peptide and, at only 12 amino-acids, was one of the smallest well-defined epitopes.

### 1.3 Insertion of DNA cassettes into the fdH gene

15 Two oligonucleotides were synthesized and purified as described in Section 1.1. One was SEQ ID No. 7, encoding (NANP)<sub>3</sub>, and the other was the complementary sequence (SEQ ID No. 8). To produce the double-stranded DNA cassette, equal amounts of these two oligonucleotides were heated to 67°C and allowed to cool slowly to room temperature. The 20 36 bp cassette was ligated into pKfdH DNA which had been treated with HpaI and calf intestine alkaline phosphatase. Ligated plasmids were transformed into E. coli strain JM109 (lacIq, rec 0). Recombinants could first be identified by restriction analysis since successful insertion of DNA 25 would destroy the HpaI site. Plasmids which were found to be resistant to digestion with HpaI were sequenced in order to determine the number and orientation of inserted cassettes. A clone was isolated that contained one copy of the 36 bp cassette in the correct orientation for 30 expression and was designated pKfdMal.

### 1.4 Expression of the fd fusion protein fdMal in vivo

E. coli cells (strain JM109) harbouring plasmid pKfdMal are induced with IPTG exactly as described for cells harbouring pKfdH (Section 1.2). Before induction 35 only E. coli proteins were present; after induction, increasing amounts of a protein appeared which was considerably larger than the wild-type fd coat protein.

Estimation of the molecular weight of the protein from polyacrylamide gel electrophoresis (approx. 7 kDa) was consistent with it being the fdMal fusion protein. Moreover, this result strongly suggested that the procoat fusion protein had been inserted into the cell membrane and correctly processed. Western-blotting of proteins from the induced cells revealed that the fusion protein was not recognised by antibodies raised against wild-type fd phage.

As a result, a general scheme for the production of fusion proteins in *E. coli* has been created; a gene containing an additional 36 base-pairs has been successfully cloned and expressed. The fusion protein contains twelve amino-acids inserted into the centre of the procoat protein (a 16% increase in size of the mature region), but these extra residues did not interfere with membrane-insertion of the protein. The leader-peptide was efficiently removed from membrane-bound procoat, indicating that the inserted epitope did not disrupt the leader-peptidase recognition sequence. Antibodies raised against wild-type fd phage did not recognise the fusion protein, suggesting that this system allows immunogenic specificity.

It is known from previous work that fd coat protein expressed from a plasmid in a non-suppressor strain of *E. coli* can support the growth of a bacteriophage, R252, that has an amber mutation in its own coat protein gene. In addition, it has been possible to produce 'hybrid' bacteriophage containing both wild-type coat proteins expressed from the phage genome and mutant coat proteins expressed from a plasmid within the host cell. This was possible even when the mutant coat protein could not support phage growth in the absence of wild-type coat protein. It has, therefore, been possible to study the ability of the fdMal coat protein to assemble into bacteriophage particles both in isolation and when 'diluted' with wild-type coat proteins.

#### 1.5 Production of hybrid bacteriophages



E. coli strain JM109 (lacIq, su) transformed with plasmid pKfdMal was grown at 37°C with shaking in YT medium containing ampicillin (50 µg/ml) until a A<sub>600</sub> value of -0.3 was reached. The cells were then infected with either  
5 phage fd or phage R252, growth was continued for 20 min and cells were induced with IPTG (10 µM final concentration). Growth was allowed to continue overnight. Phage were harvested and purified as described by Greenwood et al (1991) supra, and samples were analysed by tricine-PAGE  
10 followed by staining with silver.

The yield of R252 phage was very poor and electrophoretic analysis revealed that the only coat protein present was of the same mobility as wild-type coat protein. The small amount of phage produced was therefore  
15 assumed to be due to the low level of reversion and pseudoreversion that occurs within the population of amber mutant phages.

The yield of fd phage, on the other hand, was good (approx. 10 mg purified phage from 1 l of culture).  
20 Electrophoretic analysis revealed the presence of two coat proteins, one with the same mobility as wild-type and one with the same mobility as the fdMal protein expressed from pKfdMal. These two proteins were present in almost equal amounts (there is only slightly more of the wild-type coat  
25 protein).

#### 1.6 Characterisation of the fdMal protein

For absolute confirmation of the identity of the larger protein, a sample was separated from the wild-type protein by electrophoresis and was transferred onto PVDF  
30 membrane (method B). The N-terminal sequence was determined as SEQ ID No. 9, i.e. AEV(NANP)<sub>3</sub>N. This indicated that the complete peptide had been inserted into the expected position in the coat protein and that normal processing of the procoat had occurred.

35 It was therefore apparent that the addition of twelve amino-acids into the N-terminal domain of the mature coat protein of the phage was incompatible with phage assembly

unless the wild-type coat protein was also present. The most likely explanation for this is that the extra peptide is too bulky to allow acceptable packing of the coat proteins where the fusion proteins alone constitute the coat; however, when wild-type coat proteins are also present, these may act as 'spacers' allowing the fusion proteins to be incorporated. This has been represented diagrammatically in Figure 1. Although this adds slightly to the complexity of making phage containing fusion proteins (since it would clearly be futile to try to insert the oligonucleotide cassette directly into the phage genome), it may, in fact, be an advantage for the production of antibodies or other biologically-active peptide inserts, since the peptide is allowed more space to fold into the correct 3-dimensional structure, facilitating the production of an immune or other specific biological response.

There a number of important criteria for the successful use of the fdMal hybrid phages in vaccines. For example, it is necessary for a good immune response to be achieved without the addition of stimulants, such as Freund's Adjuvant or aluminium hydroxide, if the phage are to be injected into humans. It is important that the malaria epitope within the coat protein be available to the immune-system and that any antibodies produced against the fusion protein can also recognise the epitope when presented in a different form, such as the free peptide in solution (and eventually as part of the malaria parasite). Finally, it is important that the antibodies react specifically with the malaria epitope.

#### 1.7 Production of antibodies

New Zealand White rabbits were injected subcutaneously with 0.5 mg samples, at 1 mg/ml, of either wild-type bacteriophage fd or wild-type/fdMal hybrid bacteriophages. Rabbits were injected on days 1, 14, 28, 38, 42, 56 and 66. No other compounds were administered. After 6 weeks the rabbits were bled out and the serum was isolated.

### 1.8 Western blot analysis

Samples of wild-type phage and wild-type/fdMal hybrid phage were subjected to tricine-PAGE and were electrophoretically transferred to nitrocellulose. Western blots were prepared using either anti-fd or anti-fd/fdMal antibodies. The anti-fd antibodies strongly recognised the wild-type fd coat protein but did not recognise the fdMal protein. Anti-fd/fdMal antibodies recognised both the fd protein and the fdMal protein. This was expected since both proteins are present in the hybrid phage which were injected into rabbits. However, the reaction against the fdMal protein was noticeably stronger than the reaction against the wild-type protein. This suggested two things: (1) that the larger fusion proteins were either more antigenic than the fd coat proteins or that they were shielding the wild-type proteins from the immune-system, so that there were more antibodies produced against fdMal than against fd; (2) that the anti-fdMal antibodies were not recognising the wild-type coat proteins. Both of these factors suggested that the hybrid phage had generated a response predominantly directed against the malaria epitope attached to the phage rather than the phage themselves.

### 1.9 Recognition of synthetic peptides

The antibodies generated against the fd/fdMal hybrid phage clearly reacted against the fdMal fusion protein; however, it was important that it could also recognise the malaria epitope when it was presented in a different form. The easiest way of testing for this was to check reactivity against an (NANP)<sub>3</sub> peptide that had been chemically synthesized.

In fact, synthesis of (NANP)<sub>3</sub> itself is technically difficult because the C-terminal proline residue causes premature termination. To circumvent these difficulties and extra amino-acid (asn = N) was added to the C-terminal end. Samples of purified (NANP)<sub>3</sub>N (see SEQ ID No. 10) were spotted onto nitrocellulose and were allowed to dry. For comparison, a second unrelated peptide (SEQ ID No. 11), a

sample of phage fd and a sample of phage fd/fdMal were treated in a similar fashion. These samples were incubated with either anti-fd antibody (1:1000 dilution) or anti-fd/fdMal antibody (at dilutions of 1:500, 1:1000, 1:2000, 1:5000). After washing the filters, goat anti-rabbit IgG-alkaline phosphatase-conjugate was added. Following further washing, the filters were developed by addition of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

The antibodies raised against bacteriophage fd reacted with both the fd and fd/fdMal hybrid phage samples. These antibodies did not cross-react at all with either the synthetic (NANP)<sub>3</sub>N peptide or with the control peptide. Antibodies raised against fd/fdMal hybrids, however, reacted not only with both pure and hybrid phage samples, but also very strongly with the synthetic (NANP)<sub>3</sub>N peptide. No reaction was seen with the control peptide.

Injection of hybrid bacteriophage into rabbits, without the addition of an immunostimulant, such as Freund's Complete Adjuvant, was sufficient to generate a strong immune-response. A high titre of antibodies was produced and these reacted strongly with a sample of hybrid bacteriophage. The mixed population of antibodies had a great affinity for the malaria fusion protein, could recognise a synthetic version of the malaria peptide and did not cross-react with an unrelated peptide. Hence, the immune-response was both vigorous and very specific.

In summary of Example 1, a malaria parasite peptide has been inserted into the protein capsid of bacteriophage fd; the vigorous production of antibodies in rabbits and the ability of the antibodies to cross-react strongly with a synthetic malaria peptide have been demonstrated.

#### Example 2

In this Example, oligonucleotides were designed and subcloned into gene VIII DNA to enable a region of the 141-160 epitope from FMDV Vp1 to be displayed on the outside of the phage particles.

## 2.1 Making fd/fd FMDV hybrid bacteriophage

Oligonucleotide cassettes (SEQ ID Nos. 12 and 13) were synthesised. These encode the region 141-154 in FMDV Vp1. The oligonucleotides were treated with the enzyme polynucleotide kinase, annealed and ligated into pKfdH (as previously described). Plasmids containing the cassettes in the correct orientation were screened for by DNA sequencing. Hybrid bacteriophage were made (as described above) which contained a ratio of wild-type to extended coat protein of 3:1. Rabbits were inoculated with these bacteriophage; serum was isolated and assayed using an ELISA system. Either bacteriophage at 0.1 µg/ml or chemically-synthesised peptide SEQ ID No. 14 cross-linked to keyhole limpet haemocyanin was adsorbed onto 96-well plates in 0.1M sodium carbonate buffer, pH 8.3. After 16 hours at room temperature the unoccupied sites were blocked by the addition of 5% bovine serum albumin in Tris-buffered saline and 0.1% Tween 20 (TBST) for 2 hours at room temperature. The rabbit serum was then added to the plates at a range of dilutions.

After 30 min at room temperature, the serum was removed and the plates washed with TBST. The plates were incubated for 30 min with goat anti-rabbit IgG cross-linked to alkaline phosphatase at 1:1000 dilution. The plates were washed to remove excess secondary antibody and the presence of the alkaline phosphatase located using an AMPAK system (Novo Bio).

The rabbit serum was found to react strongly with both the hybrid bacteriophage and the chemically-synthesised peptide, showing that the rabbit was responding to the epitopes displayed on the outside of the bacteriophage.

## 2.2 Testing the fd/FMDV bacteriophage as a vaccine

Guinea pigs (4 in each of 5 groups plus control animals) were inoculated with the fd/FMDV hybrid bacteriophage and various adjuvants. The animals were then challenged with live virus.

The amounts of phage, the adjuvants and the results are given in the following Table (FICA = Freund's Incomplete Adjuvant). The results clearly illustrate that the fd/fd FMDV hybrid bacteriophage provide protection  
5 against FMDV.

	Dose	Adjuvant	Protection	Lesion
10	0	-	0/4	12
	500 µg	PBS	2/4	2
15	500 µg	FICA	4/4	0
	500 µg	Al(OH) <sub>3</sub>	4/4	0
20	50 µg	FICA	2/4	5
25	50 µg	Al(OH) <sub>3</sub>	1/4	8

In summary, Example 2 demonstrates that, by displaying epitopes on the surface of bacteriophage, it is possible to create an highly effective vaccine to FMDV. This vaccine  
30 has many powerful advantages over those that are already commercially available. It is very stable and does not require refrigeration. It is extremely cheap and easy to produce. Moreover, a wide range of different epitopes can be displayed on the surface of the bacteriophage to create  
35 a vaccine that can protect against number of serotypes. This technology can be used to develop vaccines against numerous infectious diseases including AIDS.

#### Example 3

A unique HpaI-restriction site was engineered between  
40 the codons specifying residues 3 and 4 of the mature coat protein (to generate vector fdH), and an oligonucleotide

encoding the hexapeptide YGFWGM (SEQ. ID No. 15) was cloned at this site into the bacteriophage fdH RF DNA. Viable progeny were isolated in all cases. On SDS-polyacrylamide gel electrophoresis, the lowered mobility of the enlarged coat protein was consistent with the insertion of an additional 6-amino-acids, and its amino-acid composition was found to be similarly consistent. However, it did not prove possible to insert more than 6 amino-acids directly into the major coat protein of bacteriophage fd by this technique. This might be due to one or more of several reasons: instability of the modified protein, a failure of membrane-insertion and processing of the novel procoat molecule, or an inability subsequently to package the DNA.

On the basis of the foregoing, inventors Perham, Willis and Greenwood have discovered the following subject matter that may form the subject of patent claims in the USA at least, in a continuation or continuation-in-part of Application Serial No. 07/958,321, filed December 22, 1992:

a filamentous bacteriophage (which may be degraded) including, in at least a proportion of its major coat protein, a foreign peptide of at least 6, e.g. at least 9, amino-acids that is capable of eliciting a biological response, e.g. as an agonist or antagonist of a cell receptor or an enzyme inhibitor by being antigenic or immunogenic, e.g. by raising a response against malaria or foot-and-mouth disease; a vaccine composition comprising an effective amount of a bacteriophage as defined above, in association with a physiologically-acceptable carrier or diluent;

the use of a bacteriophage as defined above for raising an immune response in a human or animal subject, by administering thereto an immune response-raising effective amount of the bacteriophage;

a method for preparing a bacteriophage as defined above, which comprises introducing a unique restriction enzyme site into gene VIII, subcloning the thus modified gene VIII into a controllable expression vector, inserting

a cassette encoding the peptide into the vector, and assembling the protein product of the resultant vector into the wild-type bacteriophage, e.g. wherein the gene encoding the engineered coat protein is transplanted into a replicon including a packaging signal.

The subject matter defined above is the subject of WO-A-9207077.

#### Background of Further Invention

The development of an anti-human immunodeficiency virus (HIV) vaccine would be a crucial step forward in combating the spread of AIDS. Many approaches to this have been explored, prominent among them being methods based on the use of the viral envelope glycoprotein gp160 and its cleavage products gp120 and gp41. The principal neutralising determinant of the virus is an intrachain disulphide-bridged loop, designated V3, in the third hypervariable region of gp120.

#### Summary of Further Invention

One aspect of the present invention is based on the discovery that peptide sequences from the V3 loop of HIV-1 strain MN (HIV-1<sub>MN</sub>) encoded in multiple display as part of the major coat protein of filamentous bacteriophage particles, are remarkably effective structural mimics of the natural epitope. They are recognised by human HIV antisera and they evoke high titres of antibodies in mice, which cross-react with other strains of HIV and are capable of neutralising the virus.

A further aspect of this invention is based on the discovery that antibody production is stimulated by simultaneous inoculation with T-cell epitopes similarly displayed on filamentous bacteriophage. The bacteriophage display system is thus a powerful means of studying the immunological recognition of HIV and a vaccine model.

Accordingly, the novel modification provides display of T-cell epitopes instead of, or in addition to, B-cell epitopes. In each case, the phage may be formulated as a vaccine, for therapeutic use, or it may be used in diagnosis.



In a particular aspect of the invention, one or different phages are engineered to display T-cell and B-cell epitopes, e.g. anti-HIV. This combination has been found to enhance the immunological response, perhaps by stimulating humeral circulating antibodies. Different phages for this purpose may be formulated as a kit.

Modified phages according to this invention may be prepared by the techniques generally and specifically described above and in WO-A-9207077, the contents of which are incorporated herein by reference.

#### Description of Further Invention

Peptides engineered genetically into the exposed N-terminal region of the major coat protein (gVIIIp) of filamentous bacteriophage fd are strongly immunogenic, even without added adjuvant. The antibodies produced are highly specific and the immune response is T-cell dependent, the phage particles themselves being able to recruit T-cell help. Synthetic peptides representing V3 loop sequences, when coupled to appropriate carrier molecules, have been shown to elicit antibodies capable of neutralising HIV and to be targets for cytotoxic T-lymphocytes and antibody-dependent T-cell mediated immunity. A major feature of the 36-residue V3 loop sequence is a relatively conserved GPGR motif at its tip, flanked by residues that differ between various HIV-1 isolates. <sup>1</sup>H-NMR studies of related synthetic peptides indicate that the GPGR motif is likely to adopt the predicted conformation of a well defined  $\beta$ -turn. We therefore selected the sequence IHIGPGRAFYTT, representing the motif in HIV-1 isolate MN, for display. See the following Table 1:

TABLE 1

Bacteriophage Coat Protein	N-terminal Sequence
fd (wild-type)	AEGDDP-
fdH	AEVNDP-
FdMN	AEV <u>I</u> HIGPGRAFYTNDP-
fdT1	AEVKQIINMWQEVGKAMYANDP-
fdESVQIN	AEVESVQINNDP-

Table 1 shows amino-acid sequences of the N-terminal regions of the wild-type and modified major coat proteins of filamentous bacteriophage fd. The inserted peptide sequences are underlined.

Oligonucleotides encoding this selected sequence were synthesised and cloned into the HpaI site of plasmid pKfdH, which was then used to construct the hybrid bacteriophage particle. It was evident from SDS-polyacrylamide gel electrophoresis of the bacteriophage coat proteins that approximately one-third of the gVIIIp molecules were carrying the peptide insert. Given that there are 2700 copies of gVIIIp per phage particle (for a review see Model and Russell, in The Bacteriophages, ed. Callender, 2:375-456 (1988), there are approximately 900 MN gp120 determinants displayed on each hybrid virion.

Balb/c mice were inoculated with 100 µg of hybrid MN phage in phosphate-buffered saline (PBS) in the absence of any adjuvant. After a primary immunisation and three subsequent boosts each one week apart, the mice were bled and their sera examined for the presence of antibodies to native HIV-1 gp120 by immunoprecipitation of lysates from cells infected with HIV-1<sub>MN</sub>. The sera from all three mice efficiently and specifically recognised the native gp120 and its precursor gp160. Sera from all mice immunised with the hybrid MN phage also cross-reacted with gp120 from HIV-

1<sub>IIIB</sub>-infected cells, suggesting that the GP<sub>GRAF</sub> motif at the tip of the V3 loop in both strains is a major part of the epitope recognised by these antibodies.

These observations contrast with those of Nardelli *et al*, J. Immunol. 148:914-920 (1992), who used synthetic V3 peptides linked to a branched lysine core. Nardelli *et al* showed that the immune response in mice was low and proportional to the length of the peptide, and that immunisation in conjunction with complete Freund's adjuvant was required. In a further study, this requirement for adjuvant was overcome by using a more elaborate macromolecular assembly of a 24-residue V3 peptide in a tetravalent format linked to tripalmitoyl S-glycerylcysteine incorporated into a liposome or micelle. See Defoort *et al*, PNAS USA 89:3879-3883 (1992).

The sera effective in immunoprecipitation were initially unable to neutralise infection by the MN virus. However, by the 21st week after the primary inoculation, two remaining mice had developed neutralising antibodies against not only HIV-1<sub>MN</sub> but the IIIB isolate as well (see Table 2, below).

HIV-1<sub>Rutz</sub>, an African isolate from Zaire, has a different sequence, GP<sub>GRAI</sub>, in its V3 loop and is a member of the genetic sub-family D, whereas MN and IIIB belong to the sub-family B (M. Reitz, personal communication). However, the serum from one mouse immunised with the hybrid MN phage neutralised the Rutz virus with a titre similar to that measured for the other HIV-1 isolates tested (Table 2). This is in contrast with the sera from rabbits immunised with keyhole limpet haemocyanin-linked V3 peptides containing the GP<sub>GRAF</sub> motif, which neutralised viral isolates that exhibit this motif but failed to neutralise isolates with variants of it. Peptide ELISA assays performed with sera obtained at the time of the onset of neutralising activity were consistent with this pattern of immunological cross-reactivity. Thus, strong responses were obtained with peptides representing the MN

and IIIB isolates, which share the GPGRAPH motif in their V3 loops, and with a peptide representing the Rutz (GPGRAI) motif. Positive but weaker responses were also obtained with a V3 peptide from the HIV-1<sub>RF</sub> isolate, in which the GPGRAPH sequence is replaced by GPGRVI.

To test if the addition of an external adjuvant could improve the immune response, mice were immunised with the hybrid MN phage adsorbed to alum, an adjuvant approved for general use in human vaccines. The sera taken after a series of four boosts, nine weeks after the primary inoculation, were analysed for their ability to precipitate gp120 and by peptide ELISA. We observed the same pattern of immunological reactivity as that obtained without adjuvant, except that the titres increased approximately four-fold. In addition, cross-reactivity and the ability to neutralise the virus developed earlier (Table 2).

The sequence ESVQIN occurs on the N-terminal side of the V3 loop of MN gp120, corresponding to a sequence reported to increase the overall immunogenicity of V3 peptides from HIV-1<sub>IIIB</sub>. Similarly, a helper T-cell site (T1, KQIINMWQEVGKAMYA, residues 428-443) has been identified in gp120 and T-cell immunity induced by immunisation with an identical 16-residue synthetic peptide. We therefore constructed a hybrid bacteriophage displaying the T1 sequence, with approximately 30% of the gVIIIp molecules bearing the insert (Table 1), and a recombinant bacteriophage displaying the ESVQIN sequence, in which, because of the smaller size of the insert, all 2700 copies of the gVIIIp molecules per virion could carry it (Table 1).

Balb/c mice were given a primary and only one boost inoculation with a 1:1 mixture of MN hybrid phage and ESVQIN recombinant phage, without adjuvant or with alum. Their sera were found to immunoprecipitate efficient and specifically gp120 from both cellular and viral lysates. ELISA assays revealed the presence of high-titre antibodies against MN, IIIB and Rutz V3 peptides, especially in the

groups inoculated with added adjuvant. The presence of high-titre antibodies correlated with the ability of these sera to neutralise virus infection (Table 2). However, neutralisation was limited to HIV-1<sub>MN</sub>. Additional boost  
5 inoculations are probably required to develop cross-neutralisation. Similar results were obtained with an inoculum of MN and T1 hybrid phage. The earlier and enhanced antibody response after immunisation with either MN and ESQIN phage or MN and T1 phage is encouraging  
10 evidence of efficient processing in vivo of the T-cell helper epitopes inserted in the additional bacteriophage particles.

The remarkable ability of the 12-residue peptide insert to mimic the natural HIV epitope revealed by the  
15 above study was further emphasised by the results of peptide ELISA assays using the sera from two different human HIV patients. The assay with hybrid MN phage as antigen was substantially more sensitive than that with a 23-residue synthetic peptide; moreover, under the same  
20 conditions, a 12-residue peptide whose sequence was identical to that inserted in the bacteriophage coat protein was virtually inactive.

In a separate experiment, the mouse sera induced by inoculation with hybrid MN phage were found to be unable to  
25 react with gp120 blotted onto nitrocellulose after SDS-polyacrylamide gel electrophoresis with prior reduction to break disulphide bridges, suggesting that the nature structure of the V3 loop contributes to the epitope. The bacteriophage surface evidently offers an unusually  
30 sympathetic context for even a relatively small peptide insert to adopt a conformation that is close to its natural state in the V3 loop in the HIV-1 virion.

Bacteriophage display is a highly promising approach for the favourable presentation of peptide antigens to the  
35 immune system. It clearly has potential for exploring the immune response to a defined sequence epitope, and for

designing simple immunological test reagents and inexpensive, benign vaccines.

This work was supported in part by Contracts NO1-CP-73723 and NO1-CP-73722 from the National Cancer Institute.

5 The following methods were used.

Method 1

Oligonucleotides encoding the amino-acid sequences of the peptide inserts (MN 5' - ATTCATATCGGCCCTGGCCGCGCCTTCTATAACCACG-3' and T1, 5' - AAACAGATCATCAACATGTGGCAGGAAGTTGGTAAAGCCATGTATGCC-3') were ligated into the vector pKfdH (WO-A-9207077) that had previously been digested with HpaI and then treated with calf intestinal alkaline phosphatase. Plasmids (pKfdMN and pKfdT1, respectively) containing the insert DNA in the correct orientation were identified by sequence analysis. Hybrid bacteriophage particles were generated by infecting E. coli JM109 cells transformed with the relevant plasmid with wild-type bacteriophage fd, as described in WO-A-9207077. An oligonucleotide (5'-GAATCCGTGCAGATTAAC-3') encoding the peptide insert ESVQIN ligated direct into the RF DNA of bacteriophage fdH cut with HpaI and treated with phosphatase, and the recombinant virions were prepared as described in WO-A-9207077. SDS-PAGE was carried out in 16% polyacrylamide gels and the proteins were revealed by silver-staining. The wild-type showed bands at 6.3, 9.2, 10.7 and 14.5; other lanes showed a major band greater than 6.3 and considerably less than 9.2.

Method 2

a) Three Balb/c mice were given a primary inoculation subcutaneously with 100 µg of hybrid MN phage in PBS. This was boosted 2, 3 and 4 weeks later with intraperitoneal inoculations of 100 µg of hybrid MN phage in PBS. The mice were then bled and their sera analysed by immunoprecipitation with lysates of S-labelled H9 cells infected with HIV-1<sub>MN</sub> or of S-labelled Molt3 cells infected with HIV-1<sub>IIIB</sub>. The cells were labelled with S-labelled cysteine for 6 h and the immunoprecipitates of the cell

lysates were prepared and analysed by SDS-PAGE, all as described by Pal et al, Intervirology 86:86-93 (1992).

- b) Five Balb/c mice were inoculated with the hybrid MN phage as in a), except that the antigen was mixed with alum before inoculation. Mice were bled after the third boost and the sera analysed as in a).

### Method 3

- a-d) The ELISA assays were conducted with peptides bound at 2 µg/well to Immulon microtitre plates. The peptides were: MN, YNKRKRIHIGPGRAFYTTKNIIG; IIIB, NNTRKSIRIQRGPGRAFTIGKIG; Rutz, NIRKGTHVGHVGPGRAIYTTNIIG; RF, NNTRKSITKGPGRVIYATGQIIG. Results are means of antibody titres from two or three mice for a and b and five mice for c and d. The antibody titre represents the reciprocal of the serum dilution at which an absorbance >0.2 was recorded. The preimmune sera always gave absorbances <0.1.

- e) The ELISA assays were conducted with peptides (2µg/well) or hybrid MN phage and wild-type phage fd (20 µg/well). The hybrid MN phage is equivalent to 1 µg peptide/well. The peptides were: 12-mer, IHIGPGRAFYTT; 23-mer, YNKRKRIHIGPGRAFYTTKNIIG.

- The results of antibody titre from the ELISA assays of antisera from mice immunised with the hybrid MN phage and from two human HIV patients all showed RF < IIIB < RUTZ and MN. a) Mouse serum taken 21 weeks after immunisation with MN phage without added adjuvant: c.500-10,000; b) serum taken 9 weeks after immunisation with MN phage with alum as adjuvant: c.1,000-50,000; c) serum taken 4 weeks after immunisation with mixture of hybrid MN and recombinant ESVQIN phages without adjuvant: c.300-c.30,000; and d) serum taken 4 weeks after immunisation with mixture of hybrid MN and recombinant ESVQIN phages with alum as adjuvant: c.700-c.10,000. Similar results were obtained with sera from two different human HIV-1 infected individuals.

TABLE 2

5	Neutralising activity of mice antisera against various HIV isolates				
	Phage Antigen	Adjuvant	HIV-isolate tested		
			MN	IIIB	Rutz
	Hybrid MN				
	Mouse #1 (22 weeks)	-	20	10	20
10	Mouse #2 (22 weeks)	-	20	10	ND*
	Mouse #1 (10 weeks)	+	>80	20	>10†
	Mouse #2 (10 weeks)	+	40	80	80
15	Hybrid MN plus recombinant ESVQIN				
	Mice 1-4‡ (4 weeks)	+	80	<10	<10

20

Neutralisation titres (average of two assays) are expressed as the reciprocal of the serum dilution that reduced reverse transcriptase activity by 50% compared with the preimmune serum. Each neutralisation assay was carried out one week after the relevant inoculation. Schedule of immunisation with hybrid MN phage without adjuvant; three Balb/c mice were immunised with 100 µg of hybrid MN phage in PBS and identical doses were then administered as boosts after weeks 2, 3, 4, 7, 8, 9, 17 and 21. The schedule of immunisation with hybrid MN phage adsorbed to alum as adjuvant was similar, except that the boost inoculations were administered after weeks 2, 3, 4 and 9. Schedule of immunisation with the mixture of hybrid MN and recombinant ESVQIN phages or with the mixture of hybrid MN and T1 phages: five Balb/c mice were inoculated with 100 µg of either mixture adsorbed to alum as adjuvant and an identical dose was administered as a boost after 3 weeks.

\* Not determined.  
 † This was the only dilution tested because of the limited availability of the serum. Thus it does not correspond to the end point.

‡ Average of the values for four different mice.



SEQUENCE LISTING

SEQ ID No. 1

Sequence Type: Peptide

Sequence Length: 4 amino-acids

Original Source Organism: Plasmodium falciparum

Asn Ala Asn Pro

SEQ ID No. 2

Sequence Type: Peptide

Sequence Length: 4 amino-acids

Original Source Organism: Plasmodium falciparum

Asn Asp Val Pro

SEQ ID No. 3

Sequence Type: Nucleotide

Sequence Length: 18 bases

Strandedness: Single

Topology: Linear

GGGATCGTTA ACCTCAGC

SEQ ID No. 4

Sequence Type: Nucleotide

Sequence Length: 17 bases

Strandedness: Single

Topology: Linear

CCATCGCCCA CGCATAA

## SEQ ID No. 5

Sequence Type: Peptide

Sequence Length: 9 amino-acids

Original Source Organism: Bacteriophage fdh

Feature: Ala Ala Leader-Peptidase recognition site

Ser Phe Ala Ala Glu Gly Asp Asp Pro  
-1 1 5

## SEQ ID No. 6

Sequence Type: Peptide

Sequence Length: 9 amino-acids

Original Source Organism: Bacteriophage fdh

Feature: Val Asn Insertion site

Ser Phe Ala Ala Glu Val Asn Asp Pro  
-1 1 5

## SEQ ID No. 7

Sequence Type: Nucleotide with corresponding peptide

Sequence Length: 36 bases

Strandedness: Single

Topology: Linear

AAT GCA AAC CCG AAC GCA AAC CCG AAT GCA AAC CCG  
Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro  
5 10

## SEQ ID No. 8

Sequence Type: Nucleotide

Sequence Length: 36 bases

Strandedness: Single

Topology: Linear

CGG GTT TGC ATT CGG GTT TGC GTT CGG GTT TGC ATT

**Sequence Length: 16 amino-acids**

Ala Glu Val Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn  
5 10 15

**Sequence Type:** Peptide

**Sequence Length: 13 amino-acids**

Asn Ala Asn Pro Asn Ala Asn Pro Asn Ala Asn Pro Asn  
5 10

**Sequence Type:** Peptide

**Sequence Length: 11 amino-acids**

Ala Ala Pro Ala Ala Ala Pro Ala Pro Ala Ala  
5 10

**Sequence Type:** Nucleotide

**Sequence Length: 36 bases**

**Strandedness: Single**

**Topology: Linear**

CGG CAG GGT GCG CGC CAC TTT CTG CGC CAG CAC CTG

**Sequence Type:** Nucleotide

**Sequence Length: 36 bases**

**Strandedness:** Single

**Topology: Linear**

CTG CGC CAG CAC CTG CAG ATC GCC GCG CAG GTT CGG

34

SEQ ID No. 14

Sequence Type: Peptide

Sequence Length: 19 amino-acids

Val Pro Asn Leu Arg Gly Asp Gln Val Leu Ala Gln Lys Val Ala  
5 10 15

Arg Thr Leu Pro

SEQ ID No. 15

Sequence Type: Nucleotide with corresponding peptide

Sequence Length: 18 bases

Original Source Organism: N-terminal peptide of  $\beta$ -endorphin

TAT GGT TTC TGG GGT ATG  
Tyr Gly Phe Trp Gly Met  
5

SUBSTITUTE SHEET (RULE 26)

CLAIMS

1. A filamentous bacteriophage engineered to display T-cell epitopes and B-cell epitopes.
2. A filamentous bacteriophage engineered to display T-cell epitopes and a peptide capable of eliciting HIV-neutralising antibodies.
3. A filamentous bacteriophage engineered to display a peptide capable of eliciting HIV-neutralising antibodies.
4. A filamentous bacteriophage engineered to display T-cell epitopes.
5. A bacteriophage according to any preceding claim, displaying the motif GPGR.
6. A bacteriophage according to any preceding claim, comprising a nucleotide sequence as defined herein, e.g. as shown in Table 1.
7. A bacteriophage according to any preceding claim, engineered in the major coat protein.
8. A product comprising, as a combined formulation for simultaneous, separate or sequential use in stimulating the immune system, first and second filamentous bacteriophages respectively engineered to display B-cell epitopes and T-cell epitopes.
9. A product according to claim 8, wherein the second bacteriophage is as defined in claim 2 or claim 3.
10. A vaccine composition comprising a bacteriophage according to any of claims 1 to 7.
11. A vaccine composition comprising first and second bacteriophages as defined in claim 8 or claim 9.
12. A product according to any preceding claim, wherein the or each bacteriophage is of the type defined in any claim of WO-A-9207077.

1/1

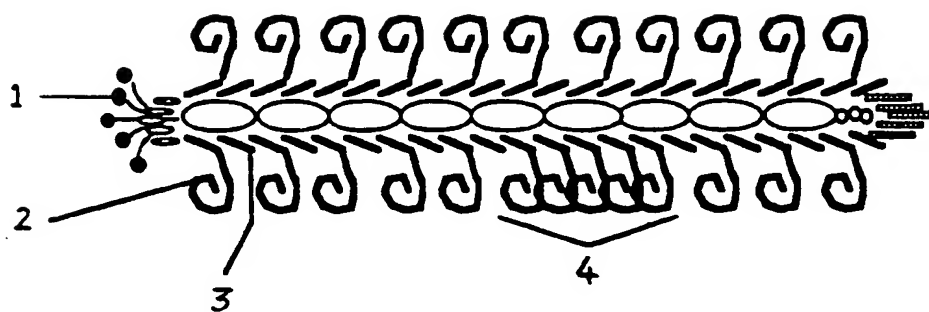


Fig. 1

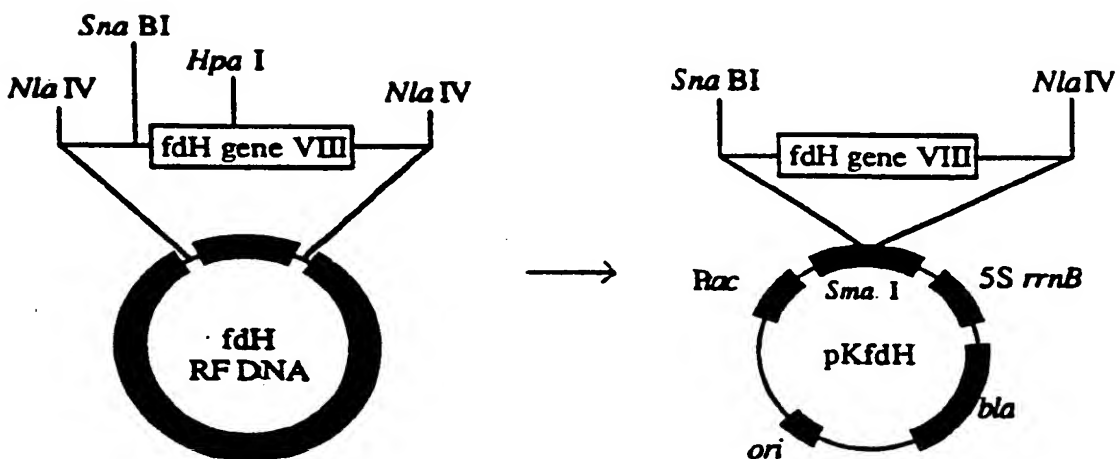


Fig. 2

SUBSTITUTE SHEET (RULE 26)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 94/01827

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 7/01, A61K 39/00, C12N 15/86 // A61K 39/21, C07K 14/155  
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB, A, 2253626 (BRITISH TECHNOLOGY GROUP PLC), 16 Sept 1992 (16.09.92), see page 4, last paragraph - page 5, first paragraph, page 8, line 26 - page 9, line 13 and example 3 --	1-6, 11, 12
A	Aids, Volume 3, No 1, 1989, Dani P. Bolognesi, "HIV antibodies and vaccine design", page 111 - page 118, see Table 1 and Fig. 1 --	1-5
X	Gene, Volume 128, 1993, O.O. Minenkova et al, "Design of specific immunogens using filamentous phage as the carrier" page 85 - page 88 --	3

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

15 December 1994

Date of mailing of the international search report

01-02-1995

Name and mailing address of the ISA/

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 94/01827

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	National Library of Medicine (NLM), file Medline, Medline accession no. 93212503, Keller PM: "Identification of HIV vaccine candidate peptides by screening random phage epitope libraries", Virology 1993 Apr; 193(2):709-16 --	3,5
X	Gene, Volume 128, 1993, Anne E. Willis et al, "Immunological properties of foreign peptides in multiple display on a filamentous bacteriophage", page 79 - page 83, see the whole document --	1-11
X	Dialog Information Service, file 154, Medline, Dialog accession No. 06508754, Medline accession No. 88153754, de la Cruz VF et al: "Immunogenicity and *epitope* mapping of foreign sequences via genetically engineered filamentous phage", J Biol Chem (UNITED STATES) Mar 25 1988, 263 (9) p 4318-22 --	1,4
X	Proc. Natl. Acad. Sci, Volume 87, 1990, (USA), R.L. Warren et al, "Method for identifying microbial antigens that stimulate specific lymphocyte responses: Application to Salmonella", page 9823 - page 9827, see "Discussion" in particular the last paragraph --	4
A	EP, A1, 0429816 (F. HOFFMANN-LA ROCHE AG), 5 June 1991 (05.06.91), page 4, line 23 - line 34 --	1-12
A	WO, A1, 9207077 (PERHAM, RICHARD, NELSON), 30 April 1992 (30.04.92), page 6, line 3 - line 10 -- -----	1-12

Form PCT/ISA/210 (continuation of second sheet) (July 1992)



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**Box I** Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- I Bacteriophage displaying T-cell as well as B-cell epitopes
- II Bacteriophages displaying T-cell epitopes
- III Combined formulation product comprising first and second bacteriophages, displaying B-cell and T-cell epitopes, respectively.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

SA 90054

Information on patent family members

26/11/94

International application No.

PCT/GB 94/01827

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
GB-A-	2253626	16/09/92	AU-B-	653387	29/09/94
			AU-A-	1173792	27/08/92
			EP-A-	0572433	08/12/93
			JP-T-	6504907	09/06/94
			WO-A-	9213081	06/08/92
			WO-A-	9305330	18/03/93
EP-A1-	0429816	05/06/91	AU-B-	637841	10/06/93
			AU-A-	6557190	09/05/91
			JP-A-	3173830	29/07/91
WO-A1-	9207077	30/04/92	EP-A-	0552267	28/07/93
			JP-T-	6501848	03/03/94